

# Assessment of T<sub>h</sub>1/T<sub>h</sub>2 cytokines among patients with Middle East respiratory syndrome coronavirus infection

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## Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) is a member of the beta-coronavirus genus of zoonotic origin that emerged in the Arabian Peninsula and is associated with significant morbidity and mortality. This study was conducted to assess the plasma levels of cytokines to evaluate the T<sub>h</sub>1/T<sub>h</sub>2 status among 46 MERS-CoV-infected patients (19 asymptomatic and 27 symptomatic) and 52 normal healthy controls using a customized luminex kit. Comparative analysis of data between MERS-CoV-infected patients and normal healthy controls revealed that although no difference was observed between asymptomatic MERS-CoV patients and controls, the mean plasma levels of interleukin (IL)-10 ( $44.69 \pm 40.04$  pg ml<sup>-1</sup> versus  $14.84 \pm 6.96$  pg ml<sup>-1</sup>;  $P < 0.0001$ ), IL-4 ( $22.46 \pm 8.02$  pg ml<sup>-1</sup> versus  $16.01 \pm 9.97$  pg ml<sup>-1</sup>;  $P < 0.0001$ ), IL-5 ( $10.78 \pm 2.86$  pg ml<sup>-1</sup> versus  $8.06 \pm 1.41$  pg ml<sup>-1</sup>;  $P < 0.0001$ ) and IL-13 ( $14.51 \pm 3.97$  pg ml<sup>-1</sup> versus  $11.53 \pm 4.16$  pg ml<sup>-1</sup>;  $P < 0.003$ ) in MERS-CoV symptomatic patients were significantly higher than the normal controls. The mean plasma levels of interferon (IFN)- $\gamma$  and IL-12 were no different among the study groups. The cytokine profile among symptomatic MERS-CoV-infected patients was skewed to a T<sub>h</sub>2 type immune response.

Keywords: IFN- $\gamma$ , IL-4, IL-10, IL-12, MERS-CoV

## Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) is a member of the beta-coronavirus genus that emerged in the Middle East region in 2012. This virus causes severe respiratory disease with significant mortality especially in people with co-morbidities such as chronic lung, heart and kidney diseases, diabetes, and hypertension. MERS-CoV is a zoonotic disease with the original source thought to be bats and the intermediate reservoir dromedary camels (1). However, to date, the detailed mechanism of animal-to-human and human-to-human transmission is unclear. Despite the fact that MERS-CoV has been prevalent in the Arabian Peninsula since 2012 with international spread to 27 countries, there is still no approved vaccine or effective therapy currently

available. The clinical course of MERS-CoV infection is highly variable with asymptomatic/mild cases to severe disease with multiorgan failure and the exact mechanisms involved in the pathogenesis remain obscure (1). Both innate and adaptive immune responses are believed to play a critical role in the pathogenesis of MERS-CoV infection (2). Among several immunological responses induced during MERS-CoV infection, cytokine production is considered to be a key factor influencing the clinical outcome (2).

Traditionally, T-helper lymphocytes are classified into T<sub>h</sub>1 and T<sub>h</sub>2 subsets based on the pattern of cytokine production (3, 4). The T<sub>h</sub>1 subset characteristically produces interleukin (IL)-12 and interferon (IFN)- $\gamma$  for the promotion

of cell-mediated immune responses linked with virus clearance (5). The  $T_h2$  lymphocyte subset, on the other hand, produces IL-4, IL-10 and IL-13 to promote the humoral immune response that is associated with chronicity and progression of viral infection (6, 7). These findings indicate that an imbalance between  $T_h1$  and  $T_h2$  cytokine secretion is critical in determining the outcome of MERS-CoV infection. Although  $T_h1$ - or  $T_h2$ -skewed immune responses have been described in different viral infections, the issue remains unclear in MERS-CoV infection because of conflicting reports.

## Methods

### Characteristics of the study populations

This was a cross-sectional study to assess the levels of  $T_h1/T_h2$  cytokines among MERS-CoV patients compared with healthy controls. A total of 46 (22 males and 24 females) MERS-CoV-infected patients were enrolled. The mean age of the patients was  $50.9 \pm 17.7$  years (range 19–93 years). Nineteen patients were asymptomatic, whereas 27 patients had symptoms. The predominant symptom among the patients was fever in 19 (41.3%) patients followed by cough in 10 (21.7%). The most frequent comorbidity was diabetes mellitus in eight (17.4%) patients followed by hypertension in four (8.7%) (Table 1). Blood samples were collected during the acute phase of the infection between January 2018 and September 2019. Diagnosis of MERS-CoV infection was confirmed by RT-PCR for both *upE* and *orf1a* genes of MERS-CoV. None of the patients was receiving any anti-viral or immunosuppressive medications at the time of collection of blood samples. A group of 52 (51 males and 1 female; mean age  $38.1 \pm 13.7$  years) normal healthy individuals was also included in the study. All the healthy individuals were screened and tested negative for hepatitis B or C, HIV and human T-cell leukemia virus (HTLV). This study was approved by the College of Medicine Institutional Review Board (IRB), and all patients signed an informed consent.

### Measurement of $T_h1$ and $T_h2$ cytokines

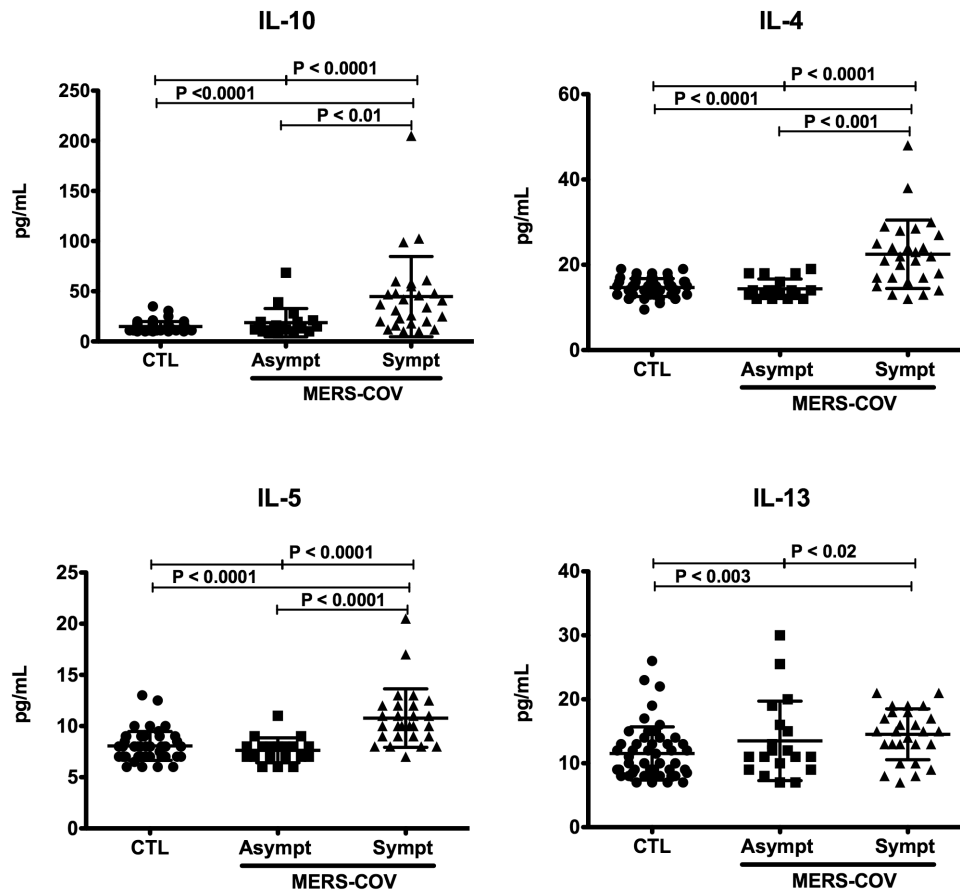
The plasma cytokine levels of IFN- $\gamma$ , IL-12, IL-2, IL-10, IL-13, IL-4 and IL-5 among patients and controls were measured by a luminex customized  $T_h1/T_h2$  human cytokine magnetic plex panel (Novex Life Technologies, CA, USA) in accordance with the manufacturer's instructions. Briefly, the human cytokine standard 1 and standard 2 were prepared by mixing 500  $\mu$ l of assay diluent to lyophilized cytokine standard and incubated for 15 min. Top standards were prepared by mixing 300  $\mu$ l of each standard followed by preparation of 1:3 serial dilutions. About 25  $\mu$ l of 1 $\times$  antibody beads were dispensed in each well of 96-well micro titer plates and incubated on a magnet for 1 min. The plate was then washed twice with 200  $\mu$ l of 1 $\times$  washing solution. After washing, 50  $\mu$ l of incubation buffer was added to all wells and 50  $\mu$ l of assay diluent and 50  $\mu$ l of plasma samples were added to all sample wells and 100  $\mu$ l of each standard to the standard wells. The plate was then covered and incubated overnight on a shaker in the dark at 4°C. Following overnight incubation, plates were washed twice with 200  $\mu$ l of 1 $\times$  washing solution, and 100  $\mu$ l of 1 $\times$  biotinylated detector antibody was added and

**Table 1.** Demographic and clinical details of MERS-CoV-positive patients

Patient #	Gender	Age	Signs and symptoms
1	M	19	Asymptomatic
2	F	25	Common cold
3	M	64	Fever
4	M	61	Fever, fatigue, nausea
5	F	36	Sore throat
6	F	38	Asymptomatic
7	F	35	Asymptomatic
8	F	41	Asymptomatic
9	M	66	Confusion, fever
10	F	58	Asymptomatic
11	F	69	Pneumonia
12	F	64	Asymptomatic
13	F	52	Fever, pneumonia
14	F	54	Asymptomatic
15	F	75	Pneumonia
16	F	60	Asymptomatic
17	F	62	Asymptomatic
18	F	51	Asymptomatic
19	F	32	Asymptomatic
20	F	59	Asymptomatic
21	F	34	Asymptomatic
22	F	34	Asymptomatic
23	F	21	Asymptomatic
24	F	87	Body weakness
25	F	29	Asymptomatic
26	F	42	Asymptomatic
27	M	45	Fever, nausea
28	M	83	Cough, fever, fatigue, chest pain
29	M	45	Asymptomatic
30	F	31	Asthma
31	M	67	Asymptomatic
32	M	61	Fever, rigors, DM
33	M	44	Cough, fever, DM
34	M	40	SOB, cough, fever
35	M	53	Vomiting, nausea, SOB, cough with sputum, fever, DM, HTN, HF
36	M	53	Vomiting, nausea, cough with sputum, fever, HTN
37	M	32	Cough, fever
38	M	75	SOB, cough with sputum, DM, HTN, CAD
39	F	66	Cough, fever, DM
40	M	93	Vomiting, diarrhea, fever, fatigue, malaise
41	M	41	Fever
42	M	32	Diarrhea, SOB, fever, fatigue, malaise
43	M	35	Cough, fever
44	M	63	SOB, DM, HTN, asthma
45	M	50	SOB, cough, fever, DM
46	M	65	Fever, DM

CAD, coronary artery disease; DM, diabetes mellitus; HF, heart failure; HTN, hypertension; SOB, short of breathing.

incubated on a shaker in the dark for 1 h. The plate was then washed twice with 200  $\mu$ l of 1 $\times$  washing solution and 100  $\mu$ l of 1 $\times$  streptavidin-R-Phycoerythrin (RPE) solution was added and incubated on a shaker in the dark for 30 min. Finally, the plate was washed three times with 200  $\mu$ l of 1 $\times$  washing solution and re-suspended with 150  $\mu$ l of 1 $\times$  washing solution, shaken for 3 min and read by the luminex MAGPIX system. Interpretation of results was performed by using xPONENT



**Fig. 1.** Human  $T_h2$  cytokine levels in patient study groups. Plasma from MERS-CoV patients (symptomatic  $n = 27$  and asymptomatic  $n = 19$ ) and healthy controls (CTL) ( $n = 52$ ) was collected and cytokines were analyzed using the luminex customized  $T_h1/T_h2$  human cytokine magnetic plex panel and luminex MAGPIX system. The mean levels of IL-10, IL-13, IL-14 and IL-5 from the patients' study groups are graphed along with the standard deviation. A one-way ANOVA test was used for comparison of all the groups. An unpaired  $t$ -test was used for comparisons between two study groups and the  $P$  value is shown where it is significant.

software and the concentrations of cytokines were expressed in picograms per milliliter ( $\text{pg ml}^{-1}$ ). The analytical sensitivities for IFN- $\gamma$ , IL-2, IL-10 and IL-5 were  $<0.5 \text{ pg ml}^{-1}$  and for IL-4, IL-12 and IL-13 were  $<1 \text{ pg ml}^{-1}$ .

*RNA extraction and MERS-CoV detection using RT-PCR*

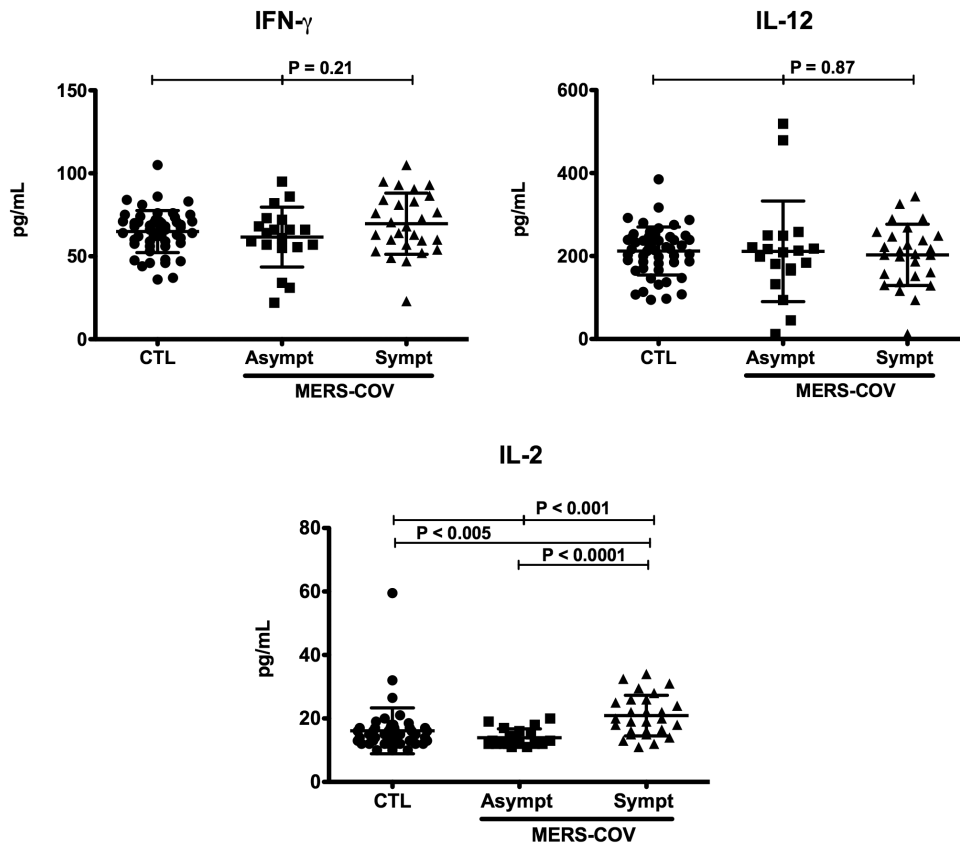
Total nucleic acid was extracted from 300  $\mu\text{l}$  of viral transport medium specimen and eluted in a final volume of 50  $\mu\text{l}$  using the Nucleic Acid Isolation Kit I and the MagNA Pure Compact system (Roche Applied Science) as instructed by the manufacturer's protocol. Then, 10  $\mu\text{l}$  of the eluted RNA was reverse transcribed to cDNA using random primers. Then, the produced cDNA was amplified for the detection of MERS-CoV *upE* gene and *orf1a* gene using specific primers and probes of the Altona kit (Hamburg, Germany), and Rotergene (Qiagen, Santa Clarita, CA, USA).

*Statistical analysis*

Data were statistically analyzed using GraphPad Prism 5 software. Parametric statistical testing among all groups was performed by one-way analysis of variance (ANOVA). Parametric statistical testing between two study groups was performed by unpaired  $t$ -test. A  $P \leq 0.05$  was considered statistically significant.

**Results and discussion**

Comparative analysis of data between MERS-CoV-infected patients and the normal healthy controls revealed  $T_h2$ -skewed immune responses among patients with MERS-CoV infection. Although no difference was evident between asymptomatic MERS-CoV patients and controls, the mean plasma levels of IL-10 ( $44.69 \pm 40.04 \text{ pg ml}^{-1}$  versus  $14.84 \pm 6.96 \text{ pg ml}^{-1}$ ;  $P < 0.0001$ ), IL-4 ( $22.46 \pm 8.02 \text{ pg ml}^{-1}$  versus  $16.01 \pm 9.97 \text{ pg ml}^{-1}$ ;  $P < 0.0001$ ), IL-5 ( $10.78 \pm 2.86 \text{ pg ml}^{-1}$  versus  $8.06 \pm 1.41 \text{ pg ml}^{-1}$ ;  $P < 0.0001$ ) and IL-13 ( $14.51 \pm 3.97 \text{ pg ml}^{-1}$  versus  $11.53 \pm 4.16 \text{ pg ml}^{-1}$ ;  $P < 0.003$ ) in MERS-CoV symptomatic patients were significantly higher than the normal controls (Fig. 1). No statistically significant differences were observed between asymptomatic MERS-CoV patients and normal healthy controls for IL-10, IL-4, IL-5 and IL-13 (Fig. 1). No statistically significant differences for  $T_h1$  cytokines such as IFN- $\gamma$  ( $69.59 \pm 18.43 \text{ pg ml}^{-1}$ ,  $61.55 \pm 18.05 \text{ pg ml}^{-1}$  and  $64.8 \pm 1.7 \text{ pg ml}^{-1}$ ) and IL-12 ( $202.78 \pm 73.87 \text{ pg ml}^{-1}$ ,  $211.26 \pm 121.26 \text{ pg ml}^{-1}$  and  $212.1 \pm 8.0 \text{ pg ml}^{-1}$ ) were observed among MERS-CoV symptomatic, asymptomatic patients and normal healthy controls, respectively (Fig. 2). The mean plasma levels of IL-2 in MERS-CoV symptomatic patients ( $20.91 \pm 6.40 \text{ pg ml}^{-1}$ ) were significantly higher than



**Fig. 2.** Human  $T_h1$  cytokine levels in patient study groups. Plasma from MERS-CoV patients (symptomatic  $n = 27$  and asymptomatic  $n = 19$ ) and healthy controls (CTL) ( $n = 52$ ) was collected and cytokines were analyzed using the luminex customized  $T_h1/T_h2$  human cytokine magnetic plex panel and luminex MAGPIX system. The mean levels of IFN- $\gamma$ , IL-12 and IL-2 from the patients' study groups are graphed along with the standard deviation. A one-way ANOVA test was used for comparison of all the groups. An unpaired  $t$ -test was used for comparisons between two study groups and the  $P$  value is shown where it is significant.

the healthy controls ( $16.10 \pm 7.21$  pg ml $^{-1}$ ,  $P < 0.005$ ) and MERS-CoV asymptomatic patients ( $13.92 \pm 2.80$  pg ml $^{-1}$ ,  $P < 0.0001$ ). The mean plasma levels of IL-2 in MERS-CoV asymptomatic patients and the normal healthy controls were no different (Fig. 2).

The cytokine profile of the symptomatic patients infected with MERS-CoV was skewed to a  $T_h2$  type immune response. This finding appeared to be somewhat unique for MERS-CoV infection when compared with other respiratory viral infections such as severe acute respiratory syndrome coronavirus (SARS-CoV) or H1N1 Influenza virus that have been shown to induce a  $T_h1$  type immune response (8, 9). Type I interferons (IFN-I; e.g. IFN- $\alpha$  and IFN- $\beta$ ) are the key cytokines that are believed to be the first line of defense against MERS-CoV infection. Blockade of IFN-I signaling has been shown to be associated with delayed virus clearance, increased neutrophil infiltration and impaired MERS-CoV responses (10). It has been shown that MERS-CoV M protein suppresses IFN-I expression by blocking the TANK-binding kinase 1 (TBK1) / interferon regulatory factor 3 (IRF-3) activation pathway (11). These observations suggest that inadequate release of IFN-I because of a  $T_h2$ -skewed cytokine profile in MERS-CoV may promote intense inflammation among MERS-CoV-infected patients. Recently, the down-regulation of both  $T_h1$  and  $T_h2$  responses reported in MERS-CoV infection has been

attributed to an upsurge of pro-inflammatory cytokines such as IL-1 $\alpha$ , IL-1 $\beta$  and IL-8 (12). In addition, an inflammatory  $T_h1$  and  $T_h17$  cytokine profile has also been reported in MERS-CoV infection recently (13). Collectively, the observations of the present study along with already published data about the cytokine profile in acute MERS-CoV infection appear to be conflicting. It is possible that MERS-CoV may have evolved multiple mechanisms to mitigate host defenses.

Interferons are major cytokines of the innate immune response against MERS-CoV infection. Dampened interferon responses because of the  $T_h2$ -skewed cytokine profile in MERS-CoV infection may therefore be critical in progression of the infection. Diminished anti-viral activity of interferons, particularly of type I and type III, in MERS-CoV infection is supported by the fact that treatment with these cytokines effectively inhibits replication of MERS-CoV (14, 15). A number of MERS-CoV proteins such as papain-like protease, membrane protein and accessory proteins, including 4a, 4b and 5, have been shown to suppress IFN-I production (16). Among the accessory proteins, 4a protein is a potent suppressor of PACT-induced activation of retinoic acid-inducible gene I (RIG-1); melanoma differentiation-associated protein 5 (MDA5), by interfering with IFN-I release, may abrogate the innate immune response against MERS-CoV (17). Moreover, MERS-CoV spike (S) glycoprotein binds to



dipeptidyl-peptidase 4 (DPP4) receptor on macrophages, resulting in a decreased production of TNF- $\alpha$  and IL-6 and augments the release of the  $T_h2$  immunosuppressive cytokine IL-10 by macrophages (18). It is, therefore, possible that MERS-CoV-induced active suppression of the  $T_h1$  response may tilt the balance in favor of a  $T_h2$  immune response.

The  $T_h2$ -skewed immune response observed in the present study could possibly be a host attempt to produce neutralizing antibodies against MERS-CoV. Experimental studies in mice have shown that immunization of mice with a vaccine containing inactivated MERS-CoV and subsequent virus challenge not only successfully induced neutralizing antibodies against MERS-CoV but also was associated with elevated serum levels of  $T_h2$  cytokines such as IL-5 and IL-13 (19). Effective antibody responses in MERS-CoV infection are critical for host survival (20). Moreover, neutralizing antibodies in serum have been shown to correlate positively with a reduction of lung inflammation and survival of hosts challenged with MERS-CoV (21). Alterations in cytokine levels in MERS-CoV infection with pneumonia occur in the second or the third week of infection (22). The inconsistent reports of the cytokine profiles among patients infected with MERS-CoV, observed in several studies, reflect an evolving pattern of the disorder and emphasize the need for further investigations.

Pregnant women appear to be disproportionately affected by respiratory illnesses and suffer from increased morbidity and mortality rates. Most human coronavirus infections are mild; however, the outcomes of past epidemics due to SARS-CoV and MERS-CoV have been grave with approximately one-third of infected pregnant women dying from the illness (23, 24). During pregnancy, a physiological shift to a  $T_h2$  immune response is observed that increases maternal susceptibility to viral infections (25) that are more effectively contained by  $T_h1$  immune responses, thus contributing to increased morbidity and mortality rates among the gravida. The findings of the present study may support the notion that  $T_h2$ -skewed immune responses in MERS-CoV infection may serve as a marker for unfavorable outcomes during the course of infection.

In conclusion, a  $T_h2$ -skewed cytokine response was observed among symptomatic patients with MERS-CoV infection. This study was limited by the lack of correlation of  $T_h2$  cytokines and disease severity because of a lack of access to patients' medical records. Data describing cytokine profiles of MERS-CoV patients are inconsistent, which could be due to variations in disease severity, presence of co-morbidities and age of the patients. Further investigations during different phases of the disorder and the associated co-morbidities may provide a better insight into the pathogenesis of MERS-CoV infection.

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